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(21) International Application Number: PCT/GB99/02806 (22) International Filing Date: 25 August 1999 (25.08.1999) (30) Priority Data: 9818548.1 25 August 1998 (25.08.1998) GB (60) Parent Application or Grant MICROBIOLOGICAL RESEARCH AUTHORITY [/]; (). QUINN, Conrad, Padraig [/]; (). FOSTER, Keith, Alan [/]; (). CHADDOCK, John, Andrew [/]; (). QUINN, Conrad, Padraig [/]; (). FOSTER, Keith, Alan [/]; (). CHADDOCK, John, Andrew [/]; (). SCHLICH, George, William ; ().		Published
(54) Title: TREATMENT OF MUCUS HYPERSECRETION (54) Titre: TRAITEMENT DE L'HYPERSECRETION DE MUCUS (57) Abstract A method of treating mucus hypersecretion, the causative factor in chronic obstructive pulmonary disease (COPD), asthma and other clinical conditions involving COPD, comprises administering a compound that inhibits exocytosis in mucus secreting cells or neurones that control or direct mucus secretion. Also described is a compound, for use in the treatment of hypersecretion of mucus, which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, and/or inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion. (57) Abrégé L'invention concerne une méthode de traitement de l'hypersécrétion de mucus, facteur causal de la bronchopneumopathie chronique obstructive (COPD), de l'asthme et d'autres états cliniques impliquant la COPD. Ladite méthode consiste à administrer un composé qui inhibe l'exocytose dans les cellules sécrétant le mucus ou les neurones qui commandent ou dirigent la sécrétion de mucus. L'invention porte également sur un composé à utiliser dans le traitement de l'hypersécrétion de mucus, qui inhibe la sécrétion de mucus en inhibant la sécrétion de mucus par les cellules sécrétant le mucus, et/ou en inhibant la libération de neurotransmetteur des cellules neuronales commandant ou dirigeant la sécrétion de mucus.		

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<p>(21) International Application Number: PCT/GB99/02806 (22) International Filing Date: 25 August 1999 (25.08.99) (30) Priority Data: 9818548.1 25 August 1998 (25.08.98) GB (71) Applicant (for all designated States except US): MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): QUINN, Conrad, Padraig [GB/GB]; Microbiological Research Authority, CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). FOSTER, Keith, Alan [GB/GB]; Microbiological Research Authority, CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). CHADDOCK, John, Andrew [GB/GB]; Microbiological Research Authority, CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB).</p>		<p>(74) Agents: SCHLICH, George, William et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB). (81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: TREATMENT OF MUCUS HYPERSECRETION</p> <p>(57) Abstract</p> <p>A method of treating mucus hypersecretion, the causative factor in chronic obstructive pulmonary disease (COPD), asthma and other clinical conditions involving COPD, comprises administering a compound that inhibits exocytosis in mucus secreting cells or neurones that control or direct mucus secretion. Also described is a compound, for use in the treatment of hypersecretion of mucus, which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, and/or inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.</p> <div data-bbox="885 1144 1291 1564"><p>2.5µg 1.3µg 0.6µg 0.3µg</p></div>		

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Description

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TREATMENT OF MUCUS HYPERSECRETION

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The present invention relates to treatment of mucus hypersecretion, to compositions therefor and manufacture of those compositions. The present invention relates particularly, though not exclusively, to the treatment of chronic bronchitis in chronic obstructive pulmonary disease (COPD), asthma and other clinical conditions involving COPD.

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Mucus is a thin film of protective viscoelastic liquid which lines the airways. It is a 1 - 2 % aqueous solution, in which the major components are the glycoconjugates known as mucins. Mucus, including the mucins, is secreted by mucus secretory cells, the surface epithelial goblet cells of the large airways and the mucus cells of the submucosal glands. Mucin release occurs by three mechanisms: constitutive secretion, regulated secretion and protease cell surface activity. Of these it is regulated secretion that responds to external stimuli and is amenable to therapeutic intervention in COPD and asthma. Regulated secretion involves release from intracellular granules by docking and fusion of the granules with the cell exterior to release their contents onto the airway surface. Fusion of the granules can either be with the plasma membrane of the epithelial cell or with the membrane of other granules leading to release via multigranular complexes fused at the cell surface. Regulated secretion of mucins is controlled by humoral factors and by neural mechanisms. The neural mechanisms in humans involve a minor contribution from the adrenergic, sympathetic pathway and a major cholinergic, parasympathetic component. Another important neural pathway regulating mucin secretion, particularly the hypersecretion of pathological conditions, is that of the Non-Adrenergic Non-Cholinergic (NANC) pathway. The NANC component involves both an orthodromic pathway involving neuropeptide and nonpeptide transmitters, and a local sensory efferent pathway involving antidromic fibres from sensory C fibres.

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COPD is a common respiratory condition, being the fourth most common cause of

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5 death in middle age in the Western world. COPD comprises two related diseases,
which usually occur together, emphysema and chronic bronchitis. The pathological
10 basis of chronic bronchitis is mucus hypersecretion. The excessive, chronic
bronchial secretion results in expectoration, and can last from a few days to many
years. The mucus hypersecretion of COPD results in small airway obstruction
producing reduced maximal respiratory flow and slow forced lung emptying. There
15 is minimal reversal of the impaired airway function of COPD by bronchodilators and
currently no effective therapy for the mucus hypersecretion.

Mucus hypersecretion is also a significant contributing factor to the
20 pathophysiology of asthma. It is a key component in *status asthmaticus*, and
contributes to the chronic symptoms and morbidity of asthma. The mucus
hypersecretion component of asthma is not well controlled by current therapies,
25 particularly in severe and chronic cases.

It would accordingly be desirable to treat, reduce or prevent the mucus
hypersecretion that causes or leads to these disease conditions.

30 Accordingly, the invention provides a method of treating mucus hypersecretion
comprising inhibiting mucus secretion by mucus secreting cells and/or inhibiting
neurotransmitter release from neuronal cells that control or direct mucus secretion.
35 The invention further provides, in a second aspect, a compound, for use in the
treatment of mucus hypersecretion, which inhibits mucus secretion by (i) inhibiting
mucus secretion by mucus secreting cells, or (ii) inhibiting neurotransmitter release
40 from neuronal cells controlling or directing mucus secretion.

An advantage of the invention is that an agent for effective treatment of mucus
45 hypersecretion and associated disease states is now provided and used, offering
a relief to sufferers where hitherto there was no such agent available.

50 The present invention thus represents a new different approach to treatment of
mucus hypersecretion by inhibiting secretory processes, namely one or other or

5 both of the mucus secretion by mucus secretory cells and the secretion of neurotransmitters regulating mucus secretion. Agents of the present invention reduce mucus secretion and/or prevent the hypersecretion of COPD and asthma,
10 and any other disease in which mucus hypersecretion is a causative element.

15 A compound of the invention typically inhibits exocytosis in mucus secreting cells or neurones that control or direct mucus secretion. This compound is administered to a patient suffering from mucus hypersecretion and inhibition of exocytosis in the cells specified results in reduction of secretion of mucus. Specific disease states caused by or exacerbated by hypersecretion are localised to the airways, and hence
20 an embodiment of the invention comprises topical administration to the airways or to a selected region or to a selected portion of the airways of a compound that inhibits exocytosis in mucus secreting cells or in neurones that control or direct mucus secretion.
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30 A compound of embodiments of the invention is a polypeptide that consists of or comprises an inhibiting domain which inhibits exocytosis in the mucus secreting cell or inhibits exocytosis in a neuronal cell, thereby directly inhibiting exocytosis of mucus or one or more mucus components or indirectly inhibiting mucus secretion by inhibiting exocytosis of neurotransmitter which would in turn lead to
35 or otherwise stimulate mucus secretion. The inhibiting domain can suitably comprise a light chain of a clostridial neurotoxin, or a fragment or variant thereof which inhibits exocytosis.

40 The compound preferably further comprises a translocating domain that translocates the inhibiting domain into the cell. This domain may comprise a H_N region of a botulinum polypeptide, or a fragment or variant thereof that translocates the inhibiting domain into the cell.
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50 The compound preferably comprises a targeting domain which binds to (i) a mucus secreting cell, or (ii) a neuronal cell controlling or directing mucus secretion. The compound is thus rendered specific for these cell types. It is also optional for the
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5 compound to be relatively non-specific but for inhibition of mucus secretion to be
achieved via targeting of the compound through choice of route of administration -
the compound is hence preferably administered to mucus secreting epithelial cells
10 in the airways, specifically in the lungs. Whilst a non-specific compound of the
invention may affect exocytosis in many cells of a wide range of types, generally
only those cells that are stimulated will be affected and these stimulated cells will
15 in typical disease states be those that are secreting mucus and contributing to
disease.

20 When present, suitable targeting domains include, but are not restricted to, a
domain selected from Substance P, VIP, β_2 adrenoreceptor agonists, gastrin
releasing peptide and calcitonin gene related peptide. The precise cells targeted in
preferred embodiments of the invention are selected from (a) cells that secrete
25 mucins, such as epithelial goblet cells and submucosal gland mucus secreting cells,
(b) cells that secrete aqueous components of mucus, such as Clara cells and serous
cells, and (c) cells that control or direct mucus secretion, such as "sensory-
efferent" C-fibres, or NANC neural system fibres. The compound may be
30 administered as a substantially pure preparation all targeted to the same cell type,
or may be a mixture of compounds targeted respectively to different cells.

35 The compound of specific embodiments of the invention comprises first, second
and third domains. The first domain is adapted to cleave one or more vesicle or
plasma-membrane associated proteins essential to exocytosis. This domain
prevents exocytosis once delivered to a targeted cell. The second domain
40 translocates the compound into the cell. This domain delivers the first domain into
the cell. The third domain binds to the target cell, ie binds to (i) a mucus secreting
cell, or (ii) a neuronal cell controlling or directing mucus secretion, and may be
referred to as a targeting moiety ("TM"). The compound may be derived from a
45 toxin and it is preferred that such a compound is free of clostridial neurotoxin and
free of any clostridial neurotoxin precursor that can be converted into toxin.
Botulinum and tetanus toxin are suitable sources of domains for the compounds of
50 the invention.

5 In use, the agent of specific embodiments of the invention has a number of discrete functions. It binds to a surface structure (the Binding Site{BS}) which is characteristic of, and has a degree of specificity for, the relevant secretory cells
10 and or neurones in the airways responsible for secretion of mucus and or regulation of said secretion. It enters the cell to which it binds by a process of endocytosis. Only certain cell surface BSs can undergo endocytosis, and preferably the BS to which the agent binds is one of these. The agent enters the cytosol, and modifies
15 components of the exocytotic machinery present in the relevant secretory cells and or neurones in the airways responsible for secretion of mucus and or regulation of said secretion.

20 Surprisingly, agents of the present invention for treatment of mucus hypersecretion can be produced by modifying a clostridial neurotoxin or fragment thereof. The clostridial neurotoxins share a common architecture of a catalytic L-chain (LC, ca
25 50 kDa) disulphide linked to a receptor binding and translocating H-chain (HC, ca 100 kDa). The HC polypeptide is considered to comprise all or part of two distinct functional domains. The carboxy-terminal half of the HC (ca 50 kDa), termed the H_C domain, is involved in the high affinity, neurospecific binding of the neurotoxin
30 to cell surface receptors on the target neuron, whilst the amino-terminal half, termed the H_N domain (ca 50 kDa), is considered to mediate the translocation of at least some portion of the neurotoxin across cellular membranes such that the functional activity of the LC is expressed within the target cell. The H_N domain also
35 has the property, under conditions of low pH, of forming ion-permeable channels in lipid membranes, this may in some manner relate to its translocation function.

40 For botulinum neurotoxin type A (BoNT/A) these domains are considered to reside within amino acid residues 872-1296 for the H_C , amino acid residues 449-871 for the H_N and residues 1-448 for the LC. Digestion with trypsin effectively degrades
45 the H_C domain of the BoNT/A to generate a non-toxic fragment designated LH_N , which is no longer able to bind to and enter neurons. The LH_N fragment so produced also has the property of enhanced solubility compared to both the parent holotoxin and the isolated LC.
50

It is therefore possible to provide functional definitions of the domains within the neurotoxin molecule, as follows:

(A) clostridial neurotoxin light chain:

A metalloprotease exhibiting high substrate specificity for vesicle and/or plasma membrane associated proteins involved in the exocytotic process. In particular, it cleaves one or more of SNAP-25, VAMP (synaptobrevin / cellubrevin) and syntaxin.

(B) clostridial neurotoxin heavy chain H_N domain:

A portion of the heavy chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell.

The domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell.

The domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH.

The domain responsible for increasing the solubility of the entire polypeptide compared to the solubility of light chain alone.

(C) clostridial neurotoxin heavy chain H_C domain.

A portion of the heavy chain which is responsible for binding of the native holotoxin to cell surface receptor(s) involved in the intoxicating action of clostridial toxin prior to internalisation of the toxin into the cell.

The identity of the cellular recognition markers for these toxins is currently not understood and no specific receptor species have yet been identified although Kozaki et al. have reported that synaptotagmin may be the receptor for botulinum

5 neurotoxin type B. It is probable that each of the neurotoxins has a different receptor.

10 By covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H_C region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), that binds to a BS on the surface of the relevant secretory cells and or neurones in the airways responsible
15 for secretion of mucus and or regulation of said secretion, a novel agent capable of inhibiting mucus secretion is produced. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment of the L-chain containing the endopeptidase activity, is covalently linked to TM which
20 can also effect internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant secretory cells and or neurones in the airways responsible for secretion of mucus and or regulation of said secretion, this also produces a novel agent capable of inhibiting
25 mucus secretion.

30 Accordingly, the invention may thus provide a compound containing a first domain equivalent to a clostridial toxin light chain and a second domain providing the functional aspects of the H_N of a clostridial toxin heavy chain, whilst lacking the functional aspects of a clostridial toxin H_C domain, and a third domain which binds
35 to the target mucus secreting or mucus secretion controlling cell.

40 For the purposes of the invention, the functional property or properties of the H_N of a clostridial toxin heavy chain that are to be exhibited by the second domain of the polypeptide of the invention is translocation of the first domain into a target cell once the compound is proximal to the target cell. References hereafter to a H_N
45 domain or to the functions of a H_N domain are references to this property or properties. The second domain is not required to exhibit other properties of the H_N domain of a clostridial toxin heavy chain. A second domain of the invention can thus be relatively insoluble but retain the translocation function of a native toxin -
50 this is of use if solubility is not essential to its administration or if necessary

5 solubility is imparted to a composition made up of that domain and one or more other components by one or more of said other components.

10 The polypeptide of the invention may be obtained by expression of a recombinant nucleic acid, preferably a DNA, and is a single polypeptide, that is to say not cleaved into separate light and heavy chain domains. The polypeptide is thus available in convenient and large quantities using recombinant techniques.

15 The first domain optionally comprises a fragment or variant of a clostridial toxin light chain. The fragment is optionally an N-terminal, or C-terminal fragment of the light chain, or is an internal fragment, so long as it substantially retains the ability to cleave the vesicle or plasma-membrane associated protein essential to exocytosis. Domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem., Vol.267, No. 21, July 1992, pages 14721-25 14729. The variant has a different peptide sequence from the light chain or from the fragment, though it too is capable of cleaving the vesicle or plasma-membrane associated protein. It is conveniently obtained by insertion, deletion and/or substitution of a light chain or fragment thereof. In embodiments of the invention described below a variant sequence comprises (i) an N-terminal extension to a clostridial toxin light chain or fragment (ii) a clostridial toxin light chain or fragment modified by alteration of at least one amino acid (iii) a C-terminal extension to a clostridial toxin light chain or fragment, or (iv) combinations of 2 or more of (i)-(iii).

30 In an embodiment of the invention described in an example below, the toxin light chain and the portion of the toxin heavy chain are of botulinum toxin type A. In a further embodiment of the invention described in an example below, the toxin light chain and the portion of the toxin heavy chain are of botulinum toxin type B. 40 The polypeptide optionally comprises a light chain or fragment or variant of one toxin type and a heavy chain or fragment or variant of another toxin type.

45 In a polypeptide according to the invention said second domain preferably comprises a clostridial toxin heavy chain H_N portion or a fragment or variant of a 50

5 clostridial toxin heavy chain H_N portion. The fragment is optionally an N-terminal or C-terminal or internal fragment, so long as it retains the function of the H_N domain. Teachings of regions within the H_N responsible for its function are
10 provided for example in Biochemistry 1995, 34, pages 15175-15181 and Eur. J. Biochem, 1989, 185, pages 197-203. The variant has a different sequence from the H_N domain or fragment, though it too retains the function of the H_N domain. It is conveniently obtained by insertion, deletion and/or substitution of a H_N domain
15 or fragment thereof. In embodiments of the invention, described below, it comprises (i) an N-terminal extension to a H_N domain or fragment, (ii) a C-terminal extension to a H_N domain or fragment, (iii) a modification to a H_N domain or fragment by alteration of at least one amino acid, or (iv) combinations of 2 or more
20 of (i)-(iii). The clostridial toxin is preferably botulinum toxin or tetanus toxin.

25 These polypeptides of the invention thus typically contain two or more polypeptide first and second domain, linked by di-sulphide bridges into composite molecules, and further linked to a third domain.

30 The TM provides specificity for the BS on the relevant neuronal and or secretory cells responsible for secretion of mucus in the airways. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, F(ab)₂, Fv, ScFv, etc.), lectins, hormones, cytokines, growth factors or peptides.
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40 It is known in the art that the H_C portion of the neurotoxin molecule can be removed from the other portion of the H-chain, known as H_N, such that the H_N fragment remains disulphide linked to the L-chain of the neurotoxin providing a fragment known as LH_N. Thus, in one embodiment of the present invention the LH_N fragment of a clostridial neurotoxin is covalently linked, using linkages which may
45 include one or more spacer regions, to a TM.

50 The H_C domain of a clostridial neurotoxin may be mutated or modified, eg by chemical modification, to reduce or preferably incapacitate its ability to bind the

5 neurotoxin to receptors at the neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

10 The heavy chain of a clostridial neurotoxin, in which the H_C domain is mutated or modified, eg by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction, may be
15 combined with the L-chain of a different clostridial neurotoxin. This hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

20 In another embodiment of the invention, the H_N domain of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. This hybrid LH_N is then covalently linked, using linkages which may include one or more spacer
25 regions, to a TM. In a further embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer
30 regions, to a TM which can also effect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant secretory and/or neuronal cells in the airways responsible for secretion of mucus and or regulation of said secretion.

35 The agent is optionally expressed recombinantly as a fusion protein which includes an appropriate TM in addition to any desired spacer regions. The recombinantly
40 expressed agent may be derived wholly from the gene encoding one serotype of neurotoxin or may be a chimaera derived from genes encoding one or more serotypes. In another embodiment of the invention the required LH_N , which may be
45 a hybrid of an L and H_N from different clostridial types, is expressed recombinantly as a fusion protein with the TM, and may include one or more spacer regions

50 The light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, may be expressed recombinantly as a fusion

5 protein with a TM which can also effect the internalisation of the L-chain, or a
fragment of the L-chain containing the endopeptidase activity, into the cytoplasm
of the relevant secretory and or neuronal cells in the airways responsible for
10 secretion of mucus and or regulation of said secretion. The expressed fusion
protein may also include one or more spacer regions.

15 A neurotoxin fragment as described in the present invention can be prepared by
methods well known in the protein art, including, but not limited to, proteolytic
cleavage or by genetic engineering strategies. Said fragment is preferably a non-
toxic fragment. The conjugation may be chemical in nature using chemical or
20 covalent linkers. Conjugates according to the present invention may be prepared
by conventional methods known in the art.

25 In a third aspect, the invention provides a composition for use in treating mucus
hypersecretion, comprising
a compound according to any of the second aspect of the invention; and
at least one of a pharmaceutically acceptable excipient, adjuvant and/or
30 propellant,
wherein the composition is for administration to the airways of a patient.

35 Aerosol administration is a preferred route of administration, though the present
invention encompasses also any administration that delivers the compound to
epithelia in the airways. Nasal administration is optional though buccal is preferred.
The compound may thus be formulated for oral administration via aerosol or
40 nebuliser or as a dry powder for inhalation using conventional excipients, adjuvants
and/or propellants. The invention therefore further provides a pharmaceutical
composition comprising a compound of the invention and a pharmaceutically
acceptable carrier.
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In use the compound will generally be employed in a pharmaceutical composition
in association with a human pharmaceutical carrier, diluent and/or excipient,
50 although the exact form of the composition will depend on the mode of

5 administration. The compound may, for example, be employed in the form of an aerosol or nebulisable solution.

10 In a specific embodiment of the invention, described in further detail below, a polypeptide according to the invention comprises Substance P, and an L chain and a heavy chain H_N region of botulinum toxin A. In use, this may be administered to
15 a patient by aerosol. A solution of the polypeptide is prepared and converted into an aerosol using a nebuliser for inhalation into the lungs of nebulised particles of diameter 1-5 microns.

20 The dosage ranges for administration of the compounds of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the conjugate, the route of administration, the nature of the formulation, the age of the patient, the nature,
25 extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician. Wide variations in the required dosage, however, are to be expected depending on the precise nature of the conjugate. Variations in these dosage levels can be adjusted using standard empirical routines
30 for optimisation, as is well understood in the art.

35 Fluid unit dosage forms are prepared utilising the compound and a pyrogen-free sterile vehicle. The compound, depending on the vehicle and concentration used, can be either dissolved or suspended in the vehicle. In preparing solutions the compound can be dissolved in the vehicle, the solution being made isotonic if
40 necessary by addition of sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving. Advantageously additives such as
45 buffering, solubilising, stabilising, preservative or bactericidal, suspending or emulsifying agents and/or local anaesthetic agents may be dissolved in the vehicle.

50 Dry powders which are dissolved or suspended in a suitable vehicle prior to use

5 may be prepared by filling pre-sterilised drug substance and other ingredients into a sterile container using aseptic technique in a sterile area. Alternatively the drug and other ingredients may be dissolved into suitable containers using aseptic
10 technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

15 Compositions suitable for administration via the respiratory tract include aerosols, nebulisable solutions or microfine powders for insufflation. In the latter case, particle size of less than 50 microns, especially less than 10 microns, is preferred. Such compositions may be made up in a conventional manner and employed in
20 conjunction with conventional administration devices.

25 In further aspects of the invention, there is provided use of a compound that inhibits exocytosis in mucus secreting cells or neurones that control or direct mucus secretion in manufacture of a medicament for treating mucus hypersecretion, asthma or COPD.

30 The invention yet further provides a method of manufacture of a pharmaceutical composition, comprising:-

- obtaining a clostridial neurotoxin and modifying it so as to remove or disable its H_C portion; or
35 - obtaining a clostridial neurotoxin the H_C portion of which has been removed or disabled;
- linking the toxin with a targeting moiety that binds to (i) a mucus
40 secreting cell, or (ii) a neuronal cell that controls or directs mucus secretion.

45 The invention still further provides a method of manufacture of a pharmaceutical composition, comprising obtaining a first component having the domains:

- an inhibiting domain which inhibits exocytosis in a mucus secreting cell or neuronal cell that controls or directs mucus secretion;
- a translocating domain which translocates the inhibiting domain into
50 the cell; and

5 - linking the first component to a second component that binds to (i)
a mucus secreting cell, or (ii) a neuronal cell that controls or directs mucus
secretion.

10 The first and second components are preferably formulated in an orally
administrable composition in combination with one or more or an excipient, an
adjuvant and a propellant.

15 Specific embodiments of the invention are now illustrated in the following examples
with reference to the accompanying drawings in which:-

20 Fig. 1 illustrates the preparation of the substance P-LH_N/A conjugate
of Example 1;

 Fig. 2 shows Western blot detection of conjugated substance P-
LH_N/A;

25 Fig. 3 shows SDS-PAGE analysis of a WGA-LH_N/A purification
scheme;

 Fig.s 4-6 show inhibition of neurotransmitter release from cultured
neuronal cells; and

30 Fig. 7 shows WGA-LH_N/A inhibits release from, but does not have
specificity for, eDRG neurons.

35 **Example 1. Method for the preparation of Substance P-LHN/A Conjugates**

40 The lyophilised peptide was rehydrated in 0.1% trifluoroacetic acid (TFA) to a final
concentration of 10mM. Aliquots of this solution were stored at -20 degrees C until
use. The LH_N/A was desalted into PBSE (PBS containing 1mM EDTA). The resulting
solution (3-5mg/ml) was reacted with a three- or four-fold molar excess of SPDP
45 by addition of a 10mM stock solution of SPDP in DMSO. After 3 hours at room
temperature the reaction was terminated by desalting over a PD-10 column into
PBSE.

50 A portion of the derivatised LH_N/A was removed from the solution and reduced with

5 DTT (5mM, 30min). This sample was analysed spectrophotometrically at 280nm and 343nm to determine the degree of derivatisation. The degree of derivatisation achieved was typically 2 mol/mol.

10 The bulk of the derivatised LH_N/A and the substance P peptide were mixed in proportions such that the peptide was in four-fold molar excess. The conjugation reaction was allowed to proceed for > 16 hours at 4°C.

15 The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was applied to a PD-10 column equilibrated in PBS and protein fractions were eluted by addition of PBS. Peptide and reaction by-products eluted after the main peak of protein and were discarded.

20 The conjugate mixture was concentrated to > 1mg/ml by centrifugation through concentrators (with 10000 molecular weight exclusion limit). The concentrated conjugate mixture was analysed by SDS-PAGE and Western blotting (probed with anti-substance P antibody) to confirm linkage of substance P peptide to LH_N/A.

25 The method described is for linkage of substance P peptide covalently to LH_N/A via a SPDP linker. A sulphydryl residue is incorporated into the C-terminus of the substance P residue, in this case by the addition of a Cys residue. Alternative linkers are available, including linkers utilising similar chemistry of derivatisation but generating non-reducible covalent bonds between the linked species.

30 The Substance P peptide sequence used in this particular example is RPKPQQFFGLMC, though alternative sequences are also suitable, e.g. CRPKPQQFFGLM, ie substance P with an N-terminal Cys.

35 The method described does not make use of any tagging system (e.g. poly His) to purify the conjugate from free LH_N/A. This has been demonstrated to be a successful method for the preparation of opioid peptide LH_N/A such the receptor binding function of the peptide was not compromised. A similar approach can be

5 applied to the synthesis of subP-LH_N/A.

10 Fig. 1 illustrates the preparation of the substance P-LH_N/A conjugate of Example 1. In the results shown in Figure 1, LH_N/A and substance P-LH_N/A samples at the concentrations indicated were applied to nitrocellulose and probed with rabbit anti-substance P antibody (upper two rows). The emergence of cross-reaction with the conjugate (second row), rather than the LH_N/A (first row), is indicative of substance P conjugated to LH_N/A. The lower control row illustrates the presence of LH_N/A.

20 Figure 2 shows Western blot detection of conjugated substance P-LH_N/A. Samples of substance P-LH_N/A (lane 3) and LH_N/A (lane 2) were electrophoresed alongside molecular weight markers (lane 1). Detection of substance P by rat anti-substance P antisera indicated protein of approx. 100kDa molecular weight in the conjugate lane, but no such band in the LH_N/A only lane. Thus the conjugated LH_N/A does contain substance P.

30 Example 2. Method for the preparation of a broad specificity agent

Conjugation and purification of WGA-LH_N/A. WGA (10mg/ml in phosphate-buffered saline (PBS)) was reacted with an equal concentration of SPDP (10mM in dimethyl sulphoxide (DMSO)) for one hour at ambient temperature. Reaction by-products were removed by desalting into PBS prior to reduction of the cross-linker with dithiothreitol. The thiopyridone and DTT were then removed by desalting into PBS to result in derivatised WGA (dWGA) with 1 mole -SH incorporated per mole of WGA.

45 LH_N/A at a concentration of 3-5mg/ml in PBSE (PBS containing 1 mM EDTA) was reacted with a three or four-fold molar excess of SPDP (10 mM in DMSO). After 3 h at ambient temperature the reaction was terminated by desalting over a PD-10 column into PBSE.

50 The derivatised WGA (dWGA) and the derivatised LH_N/A (dLH_N/A) were mixed in

5 a 3:1 molar ratio. After 16 h at 4°C the mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by ultrafiltration before application to a Superose™ 12 column on an FPLC® chromatography system
10 (Pharmacia). The column was eluted with PBS and the fractions containing high molecular weight conjugate material (separated from free dWGA) were pooled and applied to PBS-washed N-acetylglucosamine-agarose (GlcNAc-agarose). WGA-LH_N/A conjugate bound to the GlcNAc-agarose and was eluted from the column by
15 the addition of 0.3M N-acetylglucosamine in PBS. The elution profile was followed at 280 nm and fractions containing conjugate were pooled, dialysed against PBS, and stored at 4°C until use.

20 Figure 3 shows SDS-PAGE analysis of WGA-LH_N/A purification scheme. Protein fractions were subjected to 4-20% polyacrylamide SDS-PAGE prior to staining with Coomassie blue. Lanes 6-8 were run in the presence of 0.1M DTT. Lanes 1 (&7)
25 and 2 (& 8) represent derivatised WGA and derivatised LH_N/A respectively. Lanes 3-5 represent conjugation mixture, post-Superose-12 chromatography and post GlcNAc-affinity chromatography respectively. Lanes 6 represents a sample of reduced final material. Approximate molecular masses (kDa) are indicated on the
30 Figure.

35 **Example 3. Preparation and maintenance of neuronal cultures and inhibition of neurotransmitter release.**

40 PC12 cells were seeded at a density of 4×10^5 cells/well onto 24 well (matrigel coated) plates (NUNC™) from stocks grown in suspension. The cells were cultured for 1 week prior to use in RPMI, 10% horse serum, 5% foetal bovine serum, 1% L-glutamine. SH-SY5Y cells were seeded at a density of 5×10^5 cells/well onto 24
45 well plates (FALCON™). The cells were cultured in HAM-F12:MEM (1:1 v/v), 15% foetal bovine serum, 1% MEM non-essential amino acids, 2mM L-glutamine for 1 week prior to use. Embryonic spinal cord (eSC) neurons were prepared from spinal cords dissected from 14-15 day old foetal Sprague Dawley rats and were used
50 after 21 days in culture using a modification of previously described method.

Inhibition of transmitter release. PC12 cells or SH-SY5Y cells were washed with a balanced salt solution (BSS: 137mM NaCl, 5mM KCl, 2mM CaCl₂, 4.2mM NaHCO₃, 1.2mM MgCl₂, 0.44mM KH₂PO₄, 5mM glucose, 20mM HEPES, pH7.4) and loaded for 1 hour with [³H]-noradrenaline (2μCi/ml, 0.5ml/well) in BSS containing 0.2mM ascorbic acid and 0.2mM pargyline. Cells were washed 4 times (at 15 minutes intervals for 1 hour) then basal release determined by a 5 minute incubation with BSS (5mM K⁺). Cells were then depolarised with 100mM K⁺ (BSS with Na⁺ reduced accordingly) for 5 minutes to determine stimulated release. Superfusate (0.5ml) was removed to tubes on ice and briefly centrifuged to pellet any detached cells. Adherent cells were solubilised in 2M acetic acid/0.1% trifluoroacetic acid (250μl/well). The quantity of released and non-released radiolabel was determined by liquid scintillation counting of cleared superfusates and cell lysates respectively. Total uptake was calculated by addition of released and retained radioactivity and the percentage release calculated ((released counts/total uptake counts) x100).

eSC neurons were loaded with [³H]-glycine for 30 minutes prior to determination of basal and potassium-stimulated release of transmitter. A sample of 0.2M NaOH-lysed cells was used to determine total counts, from which % release could be calculated.

Figures 4-6 show inhibition of neurotransmitter release from cultured neuronal cells. PC12 (Figure 4), SH-SY5Y cells (Figure 5) and eSC neurons (Figure 6) exposed for three days to a range of concentrations of WGA-LH_N/A (filled symbols) and LH_N/A (open symbols) were assessed for stimulated [³H]-noradrenaline release (SH-SY5Y and PC12 cells) or [³H]-glycine release (eSC) capability. Results are expressed as percentage inhibition compared to untreated controls. Each concentration was assessed in triplicate. For each cell type the dose response curve is representative of at least three experiments. Each point shown is the mean of at least three determinations \pm SE of the mean.

Figure 7 shows dose-response curves of WGA-LH_N/A inhibition of eDRG substance

5 P and eSC [³H]-glycine release. Cells were exposed to conjugate for three days. Representative curves are shown. Mean IC₅₀ eDRG: $0.32 \pm 0.05 \mu\text{g/ml}$ (n = 4), eSC: $0.06 \pm 0.01 \mu\text{g/ml}$ (n = 3).

10 The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for the treatment of conditions involving mucus hypersecretion, including COPD and asthma.

Claims

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CLAIMS

1. A method of treating hypersecretion of mucus, comprising administering a compound which inhibits mucus secretion by (i) inhibiting mucus secretion by mucus secreting cells, and/or (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

2. A method according to Claim 1, comprising administering a compound that inhibits exocytosis in the mucus secreting cell or neuronal cell, thereby inhibiting exocytosis of mucus or one or more mucus components or inhibiting exocytosis of neurotransmitter.

3. A method according to Claim 1 or 2, wherein the compound comprises an inhibiting domain which consists of or comprises a light chain of a clostridial neurotoxin, or a fragment or variant thereof which inhibits exocytosis.

4. A method according to Claim 3, wherein the compound comprises a targeting domain, wherein the targeting domain binds to (i) a mucus secreting cell, and/or (ii) a neuronal cell controlling or directing mucus secretion.

5. A method according to Claim 4, wherein the targeting domain comprises a domain selected from Substance P, VIP, β_2 adrenoreceptor agonists, gastrin releasing peptide and calcitonin gene related peptide.

6. A method according to any of Claims 3 to 5, wherein the compound further comprises a translocating domain that translocates the inhibiting domain into the cell.

7. A method according to Claim 6, wherein the translocating domain comprises a H_N region of a botulinum polypeptide, or a fragment or variant thereof that translocates the inhibiting domain into the cell.

5 8. A compound, for use in the treatment of hypersecretion of mucus, which
inhibits mucus secretion by (i) inhibiting mucus secretion by mucus secreting cells,
and/or (ii) inhibiting neurotransmitter release from neuronal cells controlling or
10 directing mucus secretion.

15 9. A compound according to Claim 8, which inhibits exocytosis in the mucus
secreting cell or neuronal cell, thereby inhibiting exocytosis of mucus or one or
more mucus components or inhibiting exocytosis of neurotransmitter.

20 10. A compound according to Claim 9, comprising an inhibiting domain which
consists of or comprises a light chain of a clostridial neurotoxin, or a fragment or
variant thereof which inhibits exocytosis.

25 11. A compound according to Claim 10 further comprising a targeting domain,
wherein the targeting domain binds to (i) a mucus secreting cell, and/or (ii) a
neuronal cell controlling or directing mucus secretion.

30 12. A compound according to Claim 11, wherein the targeting domain comprises
a domain selected from Substance P, VIP, beta₂ adrenoreceptor agonists, gastrin
releasing peptide and calcitonin gene related peptide.

35 13. A compound according to any of Claims 10 to 12 further comprising a
translocating domain that translocates the inhibiting domain into the cell.

40 14. A compound according to Claim 13, wherein the translocating domain
comprises a H_N region of a botulinum polypeptide, or a fragment or variant thereof
that translocates the inhibiting domain into the cell.

45 15. A composition for use in treating mucus hypersecretion, comprising
a compound according to any of Claims 8 to 14; and
at least one of an excipient, an adjuvant and a propellant,
50 wherein the composition is for administration of the compound to airways

5 of a patient.

10 16. A composition according to Claim 15, in a formulation for aerosol administration.

15 17. Use of a compound according to any of Claims 8 to 14, in manufacture of a medicament for treatment of mucus hypersecretion.

20 18. Use of a compound according to any of Claims 8 to 14, in manufacture of a medicament for treatment of asthma.

25 19. Use of a compound according to any of Claims 8 to 14, in manufacture of a medicament for treatment of chronic obstructive pulmonary disease.

30 20. A method of treating COPD, comprising administering an effective amount of a compound according to any of Claims 8 to 14 in a pharmaceutically acceptable carrier.

35 21. A method of treating asthma, comprising administering an effective amount of a compound according to any of Claims 8 to 14 in a pharmaceutically acceptable carrier.

40 22. A method of manufacture of a pharmaceutical composition, comprising:-
- obtaining a clostridial neurotoxin and modifying it so as to remove or disable its H_C portion; or
- obtaining a clostridial neurotoxin the H_C portion of which has been removed or disabled;
45 - linking the toxin with a targeting moiety that binds to (i) a mucus secreting cell, or (ii) a neuronal cell that controls or directs mucus secretion.

50 23. A method of manufacture of a pharmaceutical composition, comprising obtaining a first component having the domains:

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- an inhibiting domain which inhibits exocytosis in a mucus secreting cell or neuronal cell that controls or directs mucus secretion;

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- a translocating domain which translocates the inhibiting domain into the cell; and

15

- linking the first component to a second component that binds to (i) a mucus secreting cell, or (ii) a neuronal cell that controls or directs mucus secretion.

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24. A method according to Claim 23, comprising formulating the first and second components in an orally administrable composition in combination with one or more of an excipient, an adjuvant and a propellant.

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2.5µg 1.3µg 0.6µg 0.3µg

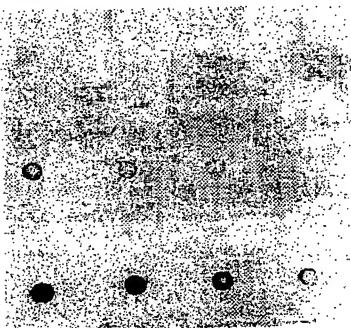


Fig. 1

1 2 3

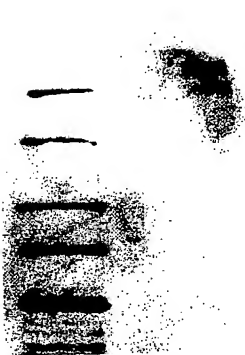


Fig. 2

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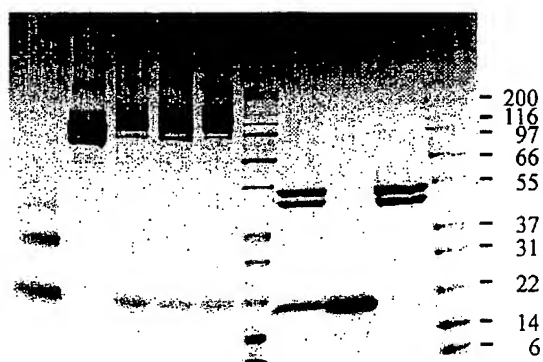


Fig. 3

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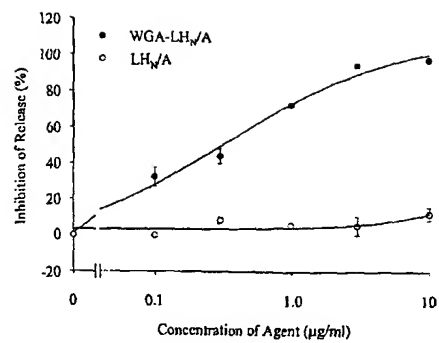


Fig. 4

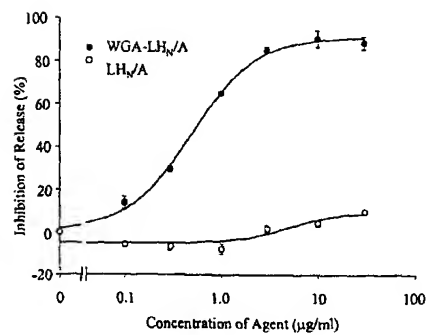


Fig. 5

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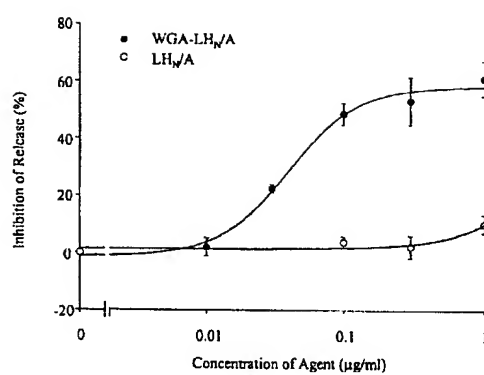


Fig. 6

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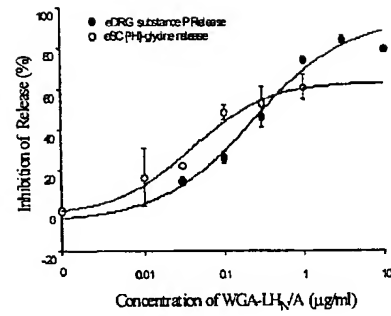


Fig. 7



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MICROBIOLOGICAL RESEARCH AUTHORITY [/];
(). QUINN, Conrad, Padraig [/]; (). FOSTER, Keith, Alan [/];
(). CHADDOCK, John, Andrew [/]; (). QUINN, Conrad,
Padraig [/]; (). FOSTER, Keith, Alan [/]; (). CHADDOCK,
John, Andrew [/]; (). SCHLICH, George, William ; ().

Published

(54) Title: RECOMBINANT BOTULINIUM TOXIN FOR THE TREATMENT OF MUCUS HYPERSECRETION
(54) Titre: TRAITEMENT DE L'HYPERSECRETION DE MUCUSEATMENT OF MUCUS HYPERSECRETION

(57) Abstract

A method of treating mucus hypersecretion, the causative factor in chronic obstructive pulmonary disease (COPD), asthma and other clinical conditions involving COPD, comprises administering a compound that inhibits exocytosis in mucus secreting cells or neurones that control or direct mucus secretion. Also described is a compound, for use in the treatment of hypersecretion of mucus, which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, and/or inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

(57) Abrégé

L'invention concerne une méthode de traitement de l'hypersécrétion de mucus, facteur causal de la bronchopneumopathie chronique obstructive (COPD), de l'asthme et d'autres états cliniques impliquant la COPD. Ladite méthode consiste à administrer un composé qui inhibe l'exocytose dans les cellules sécrétant le mucus ou les neurones qui commandent ou dirigent la sécrétion de mucus. L'invention porte également sur un composé à utiliser dans le traitement de l'hypersécrétion de mucus, qui inhibe la sécrétion de mucus en inhibant la sécrétion de mucus par les cellules sécrétant le mucus, et/ou en inhibant la libération de neurotransmetteur des cellules neuronales commandant ou dirigeant la sécrétion de mucus.

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(21) International Application Number: PCT/GB99/02806		(74) Agents: SCHLICH, George, William et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).	
(22) International Filing Date: 25 August 1999 (25.08.99)		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
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(71) Applicant (for all designated States except US): MICROBIO- LOGICAL RESEARCH AUTHORITY [GB/GB]; CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB).		(88) Date of publication of the international search report: 15 June 2000 (15.06.00)	
(72) Inventors; and (75) Inventors/Applicants (for US only): QUINN, Conrad, Padraig [GB/GB]; Microbiological Research Authority, CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). FOSTER, Keith, Alan [GB/GB]; Microbiological Research Authority, CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). CHAD- DOCK, John, Andrew [GB/GB]; Microbiological Research Authority, CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB).			
(54) Title: RECOMBINANT BOTULINIUM TOXIN FOR THE TREATMENT OF MUCUS HYPERSECRETION			
(57) Abstract <p>A method of treating mucus hypersecretion, the causative factor in chronic obstructive pulmonary disease (COPD), asthma and other clinical conditions involving COPD, comprises administering a compound that inhibits exocytosis in mucus secreting cells or neurones that control or direct mucus secretion. Also described is a compound, for use in the treatment of hypersecretion of mucus, which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, and/or inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02806

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 A61K47/48 A61P11/06 A61P11/02		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 17904 A (ALLERGAN INC) 6 July 1995 (1995-07-06)	1-3, 8-10, 15-17
Y	claims 1-5; example 5	1-24
X	WO 95 28171 A (SINAI SCHOOL MEDICINE ; SANDERS IRA (US); SHAARI CHRISTOPHER M (US)) 26 October 1995 (1995-10-26)	1-3, 6-10, 13-21
Y	the whole document	1-24
Y	WO 98 07684 A (ATOCHEM NORTH AMERICA ELF) 26 February 1998 (1998-02-26) page 4, line 25-29 page 7, line 18 -page 8, line 7 page 11, line 10-18 claims 1-9,12	1-24
-/-		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
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Date of the actual completion of the international search 2 March 2000		Date of mailing of the international search report 16/03/2000
Name and mailing address of the ISA European Patent Office, P.B. 5018 Patentstein 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3010		Authorized officer Veronese, A

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INTERNATIONAL SEARCH REPORT

Intern. nat. Application No.
PCT/GB 99/02806

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 13410 A (FISHER CAROLINE E ;MURPHY JOHN R (US); LEEMAN SUSAN E (US); BOSTON) 17 April 1997 (1997-04-17) claims; examples	1-24
Y	FISHER, CAROLINE E. ET AL: "Genetic construction and properties of a diphtheria toxin-related substance P fusion protein: in vitro destruction of cells bearing substance P receptors" PROC. NATL. ACAD. SCI. U. S. A. (1996), 93(14), 7341-7345 , XP002132007 the whole document	1-24
A	HAMBLETON P: "CLOSTRIDIUM BOTULINUM TOXINS: A GENERAL REVIEW OF INVOLVEMENT IN DISEASE, STRUCTURE, MODE OF ACTION AND PREPARATION FOR CLINICAL USE" JOURNAL OF NEUROLOGY - ZEITSCHRIFT FÜR NEUROLOGIE, DE, SPRINGER VERLAG, BERLIN, vol. 239, no. 1, 1 January 1992 (1992-01-01), pages 16-20, XP002009346 ISSN: 0340-5354	3,10
A	WO 96 33273 A (DUGGAN MICHAEL JOHN ;FOSTER KEITH ALAN (GB); MICROBIOLOGICAL RES A) 24 October 1996 (1996-10-24) the whole document	1
X	US 4 873 346 A (ANDERSON DAVID J) 10 October 1989 (1989-10-10) column 8, line 7-9 column 8, line 49-66	1,2,8,9, 15-21
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/ 02806

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-7, 20, 21
is(are) directed to a method of treatment of the human/animal
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because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
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3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/02806

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9517904 A	06-07-1995	AU 688452 B	12-03-1998
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